

Early maternal effects mediated by immunity depend on sexual ornamentation of the male partner

Nicola Saino^{1*}, Raffaella Paola Ferrari¹, Roberta Martinelli¹, Maria Romano¹, Diego Rubolini¹ and Anders Pape Møller²

¹Dipartimento di Biologia, Sez. Zoologia Scienze Naturali, Università degli Studi di Milano, via Celoria 26, I-20133 Milano, Italy

²Laboratoire d'Ecologie Evolutive Parasitaire, CNRS UMR 7103, Université Pierre et Marie Curie, 7 quai St Bernard, Case 237, F-75252 Paris Cedex 05, France

Vertebrates have an immature immune system soon after birth, and parasites can therefore be particularly virulent to young hosts. Transfer of immune factors via the egg can give rise to early maternal effects with important consequences for offspring fitness, as maternally derived immunity confers anti-parasite protection. Mothers are expected to allocate immunity differentially to the eggs according to the reproductive value of their offspring as influenced by the quality of their father. In this study, we analysed transmission to the yolk of antibodies specific to an antigen (Newcastle disease virus vaccine, NDV) by vaccinated female barn swallows (*Hirundo rustica*) mated to males whose secondary sexual characteristics had been manipulated. Concentration of anti-NDV antibodies in the yolk positively covaried with that in maternal plasma. Anti-NDV antibodies were more concentrated in the first but not the fourth eggs laid by females mated with tail-elongated males compared with those mated with tail-shortened and control males. This experiment shows that allocation of maternal immune factors to the eggs is affected by quality of the male, as signalled by its secondary sexual characteristic. Thus, early maternal effects are influenced by sexual attractiveness of male mates and are mediated by immunity.

Keywords: egg lay order; *Hirundo rustica*; immunity; maternal effects; secondary sexual characteristics

1. INTRODUCTION

Vertebrates have evolved complex immune functions to defend themselves against parasites, which can have a strong negative impact on the fitness of their hosts (Tizard 1991; Wakelin 1996; Møller 1997; Pastoret *et al.* 1998). Acquired immunity provides hosts with highly specific and efficient mechanisms to fend off parasites. However, acquired immune defences are weak and ensue slowly soon after birth (Apanius 1997; Pastoret *et al.* 1998). In addition, synchronization of a parasite reproductive cycle with that of the host (Noble & Noble 1976; Cox 1982), and particular behavioural characteristics of the host, such as coloniality, may increase offspring exposure to parasites (Loye & Zuk 1991; Clayton & Moore 1997).

Females of oviparous vertebrates deliver to their eggs components of both innate and acquired immunity. This physiological adaptation can function as an early maternal effect (Mousseau & Fox 1998). Indeed, immune factors that are contained in the egg are passed on to the embryo and, consequently, to the newborn, and may thus enhance anti-parasite defence in the crucial early life stages, compensating for poor offspring immunocompetence (Naqi *et al.* 1983; Tizard 1991; Graczyk *et al.* 1994; Smith *et al.* 1994; Lung *et al.* 1996; Ahmad & Siddique 1998; Gasperini *et al.* 2001; see also Heeb *et al.* 1998).

Resource allocation to and care of offspring involve individuals in costs in terms of survival or future repro-

duction (Lindén & Møller 1989; Clutton-Brock 1991; Stearns 1992). The theory of parental care therefore predicts that strategies should have evolved that allow differential investment in relation to the quality of the offspring, as influenced for example by the quality of their father. According to some theoretical models of sexual selection, male ornaments have evolved under the influence of female preferences for traits that reliably reveal the quality of potential mates (reviewed in Andersson (1994)). Females with a directional preference for extreme ornaments may thus accrue indirect fitness benefits in terms of inheritance of paternal genes for high viability or sexual attractiveness of the offspring. Hence, evolutionary theories of sexual selection and parental care predict that mothers should invest more in the production of offspring sired by high-quality males with large secondary sexual characteristics (Gil *et al.* 1999; Cunningham & Russell 2000, 2001; Sheldon 2000; Colegrave 2001; Gil & Graves 2001).

The aim of this study was to investigate experimentally the relationship between the expression of a secondary sexual characteristic under directional female preference (length of the outermost tail feathers) of male barn swallows (*Hirundo rustica*) and concentration of antibodies specific to a novel antigen (Newcastle disease virus vaccine, NDV) in the yolk of the eggs laid by their mates. Females were vaccinated before start of laying, while males were subjected to either tail-shortening, tail-elongation or two control treatments. In a previous study we found that anti-NDV antibodies are transmitted via the egg in this species,

* Author for correspondence (n.saino@mailserver.unimi.it).

as they were detected in the progeny of immunized females 5 days after hatching (N. Saino, unpublished results). However, concentration of antibodies in the hatchlings may be affected by protein metabolism as influenced by nutritional condition, current infection and sib-sib competition. Therefore, we measured the effect of male ornamentation on antibody transfer to the yolk in unincubated eggs. We predicted that females would transfer a larger amount of anti-NDV antibodies to the eggs when their offspring were sired by males displaying experimentally enlarged sexual ornaments.

The barn swallow is a semicolonial, socially monogamous, biparental passerine (Møller 1994). Females prefer males with long ornamental outermost tail feathers both as social and extra-pair mates (e.g. Møller 1994; Saino *et al.* 1997a). Long-tailed males are more viable, are capable of raising stronger immune responses and have smaller parasite loads than short-tailed males (Møller 1994; Saino & Møller 1996; Saino *et al.* 1997b, 1999a).

2. METHODS

We studied barn swallows in four farms east of Milano (northern Italy) during the breeding season of 2001. We captured adult swallows as soon as they started visiting the rural buildings where they breed. At first capture, adults were sexed, and length of male ornamental tail feathers was measured (Møller 1994). Each individual was given a combination of coloured leg rings and dye markings on the breast feathers that allowed assignment to nests by direct observation. A sample of blood was taken in heparinized capillary tubes. Males were assigned sequentially to one of the following tail manipulation treatments: males of the first group had their outermost tail feathers shortened by 20 mm; males of the second group were captured and subjected to morphological measurements; males of the third group had their outermost tail feathers cut and re-glued without altering their length; and the tail of males of the fourth group was elongated by 20 mm (see Møller (1994) and Saino *et al.* (1997a,b) for methodological details). Before starting the assignment, males were arranged in a random order so that treatment sequence did not reflect capture sequence. Tail length manipulation occurred three weeks on average (and never less than 10 days) before laying of the first egg by the mate of the focal males, with no significant difference in breeding stage at tail manipulation (number of days between manipulation and first egg laying) among the four groups of males (ANOVA: $F_{3,58} = 0.62$, $p = 0.61$).

Females were injected subcutaneously with 20 µl of NDV vaccine (Nobivac Paramyxio, Intervet, Milano, Italy). Blood samples were also collected 14 and 28 days post-vaccination. Plasma was then stored at -20°C for assay of anti-NDV vaccine antibodies. All females were immunized more than 9 days before laying of their first egg. Mean stage in the breeding cycle at NDV inoculation (number of days between inoculation and first egg laying) was similar in the four experimental groups (tail-shortened: 16.6 (1.41 s.e.m.); unmanipulated: 17.1 (1.46 s.e.m.); cut: 16.0 (1.06 s.e.m.); tail-elongated: 15.9 (1.45 s.e.m.); ANOVA: $F_{3,58} = 0.44$, $p = 0.72$).

Nests were inspected every day and eggs were marked. We collected the first and fourth eggs from 14 clutches of tail-shortened males, 17 clutches of unmanipulated males, 16 clutches of males with cut and re-glued tail feathers and 15 clutches of tail-elongated males. However, from four of these clutches the first

but not the fourth egg was available for immunological analyses (see figure 1). To minimize the impact on reproductive output of breeding colonies, we collected all the eggs only in a subset of the clutches from which we collected the first egg (sample size: five clutches for tail-shortened, four for unmanipulated, five for cut and five for tail-elongated males). This allowed us to check for consistency of variation in antibody concentration in the first and fourth eggs, and also in the second and third eggs. However, in the analyses we mainly focused on the first and fourth eggs. Eggs were collected on the second day after laying of the last egg, when the developing embryo is still very small, in order to be sure that clutch was complete. Each yolk was mixed and stored at -20°C .

(a) *Competitive enzyme-linked immunosorbent assay*

Anti-NDV antibody concentration was measured by monoclonal antibody-blocking enzyme-linked immunosorbent assay (ELISA) (Svanovir NDV-Ab, SVANOVA Biotech, Uppsala, Sweden) (Czifra *et al.* 1996; Nordling *et al.* 1998). Optical density (OD) values of test plasma were compared with the OD value of the kit NDV-negative control, while using plasma samples at the time of vaccination as a reference to assess the effectiveness of NDV vaccine injection in eliciting a response. OD of test yolks were compared with both the kit NDV-negative control and with a pool of yolks from uninjected females. ODs were used to calculate percentage inhibition (PI) values. PI values of yolks were significantly greater than zero when referred to the kit NDV-negative control and also to the pool of yolks ($p < 0.001$ in both cases), indicating that vaccination resulted in antibody transfer to the eggs (see also § 3). Large PI values indicate large NDV-specific blocking antibodies in the plasma and yolk samples. Clutches were assigned to ELISA plates randomly. Within-plate repeatability of OD values, as determined on different plasma and yolk samples assayed in duplicate, was 0.92 and 0.93 (ANOVA: $p < 0.003$ in both cases).

In the analyses of antibody concentration in eggs of different laying order, significance values were corrected by a sequential Bonferroni procedure for four simultaneous tests on eggs of laying order 1 to 4. *Post hoc* Bonferroni tests for differences among experimental groups are presented where needed. Observed power of non-significant statistical tests is reported.

3. RESULTS

Vaccination caused an increase in anti-NDV antibodies in females, as determined by comparing PI of antigen activity at the time of inoculation with that recorded two weeks later (increase in PI: 20.5 (2.62 s.e.m.); paired t -test; $t_{59} = 7.81$, $p < 0.0001$). Antibody concentration increased further from day 14 to day 28 of vaccination (increase in PI: 14.29 (2.39 s.e.m.); $t_{49} = 5.98$, $p < 0.0001$). Female antibody concentration two weeks after vaccination was not affected by tail manipulation of her mate (ANOVA: $F_{3,58} = 1.83$, $p = 0.15$; power = 0.452), and the same held true four weeks after vaccination (ANOVA: $F_{3,46} = 0.35$, $p = 0.79$; power = 0.112), although the power of the latter test was low and thus the non-significant result could be due to a type II statistical error. There was no significant difference in antibody levels among the experimental groups of females (repeated-measures ANOVA: between-subjects effect, $F_{3,46} = 1.42$, $p = 0.25$; power = 0.352) nor differential variation between

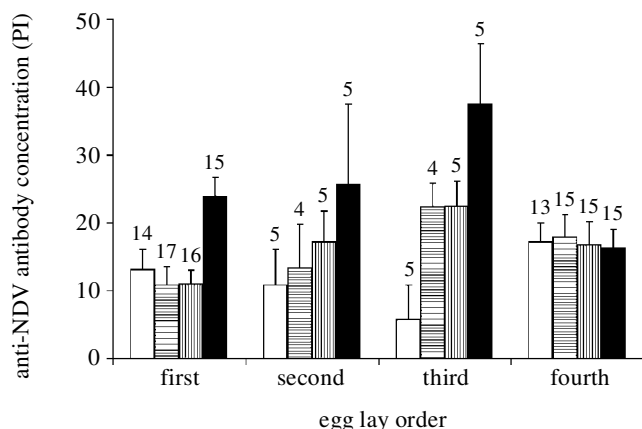


Figure 1. Mean (\pm s.e.m.) anti-NDV antibody concentration in the first to fourth eggs laid by females mated to males belonging to the four tail-manipulation treatments (white bars, shortened tail; horizontal lined bars, unmanipulated tail; vertical lined bars, cut and re-glued tail; black bars, elongated tail). Sample sizes are shown above bars. To minimize the impact on reproduction we collected second and third eggs only from a subset of the nests where we collected the first and fourth eggs. Fifth and sixth eggs were neglected because of small sample sizes.

day 14 and day 28 among male treatments ($F_{3,46} = 1.84$, $p = 0.15$; power = 0.447). Antibody concentration in maternal plasma on day 28 positively covaried with that on day 14 (ANCOVA: $F_{1,45} = 31.06$, $p < 0.0001$).

A significant effect of male tail manipulation on antibody concentration was observed in first eggs in an ANCOVA ($F_{3,57} = 5.59$, $p < 0.01$ after Bonferroni correction; figure 1), with a significant positive covariation between antibody concentration in the eggs and original tail length of males ($F_{1,57} = 8.32$, $p = 0.006$, coefficient = 0.45 (0.16 s.e.m.)). Concentration of antibodies in the first eggs of females mated to tail-elongated males was significantly larger than in each of the other groups (*post hoc* Bonferroni test: $p < 0.012$ for all comparisons). However, females mated to unmanipulated, cut or tail-shortened males had non-significantly different antibody concentration in their first egg (*post hoc* Bonferroni tests, $p > 0.99$ in all cases). We could find no significant effect of tail manipulation on antibody concentration in fourth eggs (ANCOVA: $F_{3,53} = 0.17$, $p = 0.92$, n.s., power = 0.079, figure 1; effect of original tail length: $F_{1,53} = 2.47$, $p = 0.12$, power = 0.339). Variation of antibody concentration between the first and the fourth eggs differed among tail manipulation treatments (repeated-measures ANCOVA: interaction between lay order and tail treatment: $F_{3,54} = 4.55$, $p = 0.006$).

Antibody concentration in the yolk of first eggs positively covaried with concentration in the plasma of the mother measured two weeks post-vaccination, (ANCOVA: $F_{1,56} = 5.59$, $p = 0.021$, coefficient = 0.14 (0.06 s.e.m.)), whereas there was no significant evidence for a covariation between antibody concentration in fourth eggs and anti-NDV antibodies in maternal plasma (ANCOVA: $F_{1,52} = 0.07$, $p = 0.79$; power = 0.058).

Covariation between clutch size and anti-NDV antibody concentration in the yolk of first and fourth eggs was

not statistically significant (repeated-measures ANCOVA: $t = -0.42$, $p = 0.68$).

Among second and third eggs, for which a smaller sample was available, anti-NDV antibody concentration in the eggs laid by females of tail-shortened males ranked lowest while those of tail-elongated males ranked highest. In analyses of covariance with original tail length as covariate, antibody concentration in second eggs did not vary with tail treatment ($F_{3,14} = 0.70$, $p = 0.57$, power = 0.796), whereas variation was significant among third eggs ($F_{3,14} = 4.79$, $p = 0.016$, $p < 0.05$ after Bonferroni correction). Concentration of antibodies in the third eggs of females mated to tail-elongated males was significantly larger than in the eggs of females mated to tail-shortened males (*post hoc* Bonferroni test: $p = 0.012$), whereas the other pairwise comparisons among groups of third eggs were not significant ($p > 0.36$ in all cases). All analyses described above on egg yolks were repeated using antibody concentration estimates based on negative controls consisting of a pool of yolks from unvaccinated females, and they gave qualitatively similar results.

4. DISCUSSION

In this study we have shown that concentration of maternally derived antibodies in the egg yolk of barn swallows is influenced by the expression of a secondary sexual characteristic of the male partner. First eggs of females mated with tail-elongated males contained more antibodies than those laid by females of two control groups and those mated with tail-shortened males, and a similar pattern of variation was observed in third eggs. These data were unaffected by physiological processing of yolk material during embryo development and variation of breeding stage at vaccination. The steady increase in antibody concentration until 28 days after vaccination suggests that at the time of egg laying, on average 16 days after vaccination, humoral response was still developing. The positive covariation between antibody levels in the mothers recorded 14 and 28 days post-vaccination, respectively, indicates that relative antibody levels at different times were consistent among females.

To our knowledge, this is the first experimental study showing a causal link between the expression of a secondary sexual characteristic of males and immunological quality of the eggs laid by their mates. Differential concentration of immune factors in the first eggs may enhance immune protection of offspring sired by high-quality males. In the barn swallow, first eggs are known to produce offspring that are larger and thus more viable than later eggs (Møller 1994; Saino *et al.* 2001a). Immune defence is known to affect survival in barn swallows (Saino *et al.* 1997b), suggesting that eggs and nestlings that received more antibodies were more viable.

Alternatively, a larger concentration of antibodies in the eggs of females mated to tail-elongated males may parallel variation in the offspring sex ratio associated with male ornamentation (Ellegren *et al.* 1996), as the immune profile of the offspring may have to be tuned according to their sex. In fact, variation in egg components has been shown to be related to offspring sex (Anderson *et al.* 1997; Cordero *et al.* 2000, 2001; Cunningham & Russell 2001). This interpretation, however, is unlikely because in

another experiment there was no effect of tail manipulation on progeny sex ratio (Saino *et al.* 1999b).

We found no significant evidence of variation in maternal plasma anti-NDV antibodies among male tail-manipulation treatments. Hence, females mated with the most ornamented males may have allocated their larger production of antibodies to their first eggs without increasing their own levels of immune defence. The fact that in fourth eggs no evidence of significant variation in antibody concentration existed may indicate that a physiological constraint exists on the amount of antibodies that females can transfer to consecutive eggs, making it impossible for mates of tail-elongated males to allocate additional antibodies to their last eggs. However, the power of this statistical test was low, indicating that results on fourth eggs may have been affected by a type II statistical error.

Present results indicate that females did not just passively transfer antibodies to their eggs, as this hypothesis would predict similar responses independently of male ornamentation and lay order. In addition, females did not adopt an indiscriminate allocation strategy relative to mate attractiveness, as antibody concentration depended on lay order. This indicates that females follow a fine-tuned strategy of allocation depending on male ornamentation and lay order. Because fifth and sixth eggs were rare, we can exclude the possibility that females simply allocated a fixed amount of antibody to a clutch, because females mated to tail-elongated males allocated more antibody to their clutch than any other female (figure 1).

The results of this study have obvious implications for theories of the evolution of parental investment and sexual selection. Transfer of passive immunity can have important consequences for condition and survival of offspring later on (see § 1). However, females may experience a cost of reproduction consisting of biosynthesis of antibodies, as also shown by the increase in total antibody concentration of female barn swallows prior to egg laying (Saino *et al.* 2001b). Costs of immune system functioning (Klasing & Leshchinsky 1999) and parasitism may select for the evolution of allocation strategies that allow females to invest more in immunological protection of offspring with large reproductive value. If males with large sexual ornaments are sexually attractive and/or reliably signal their desirable genetic quality, females should value their offspring more when sired by such males. In addition, the positive covariation we observed between pre-manipulation length of male tail ornaments and antibody concentration in the first eggs indicates that egg quality was influenced by original male phenotype, possibly because females responded to male traits (e.g. carotenoid-based coloration of forehead and throat feathers) that are correlated with length of ornamental tail feathers.

We conclude that early maternal effects mediated by the immune system vary in relation to the quality of male mates, as reflected by the expression of a secondary sexual characteristic, in accordance with evolutionary theory predicting larger investment in offspring with large reproductive value.

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